Final Report for the Project:

CREATING A COLLECTION OF MICROALGAE FOR USE IN BIOFUELS RESEARCH

ABSTRACT

Microalgae can either directly produce renewable fuel molecules or they can produce precursor molecules that can be processed for generation of jetfuel. The term microalgae describes a very diverse group of thousands of organisms with different metabolic properties. For this reason a variety of freshwater, brackish, marine, and hypersaline habitats was sampled at different times during the past year to isolate new miroalgae strains for future screening regarding their capabilities of production of metabolites for jetfuel production. In an 11 month effort the goal to isolate 150 new unialgal strains was achieved. About 60 more samples are at the stage of 2 to 3 microalgal strains per sample. In addition, a large number of enrichment cultures was generated that is currently being used to isolate more strains. Screening efforts are under way to identify the lipid content of novel strains.

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14. ABSTRACT
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I. OBJECTIVE

For both direct and metabolic engineering approaches to improved biofuels production, it is vital to isolate a large variety of microalgae for assembly into a culture collection serving as a bioresource for further biofuels research. Consequently, the **major objective** of this research was to create a culture collection as a resource of diverse microalgae for biofuels research. To this end, from about 50 different habitats novel microalgae strains were to be isolated. This 11 month project employed a dual spatial and temporal sampling strategy that included numerous sampling sites representing very diverse environments and also repetitive collection thus accounting for temporal succession of microalgae communities. As most current mass culture operations are based on outdoor shallow open-pond systems, sampling sites included a variety of shallow aqueous habitats of about 1 to 10 inches depth.

Pay-off to the Airforce: Microalgae can be used to produce renewable jetfuel. Isolation of a large variety of novel microalgae strains will allow screening for their capabilities to synthesize metabolites for production of renewable jetfuel.

II. RESULTS AND ACCOMPLISHMENTS

Overall the project was based on the following three major tasks:
Task 1 – Sampling
Task 2 – Isolation of Strains
Task 3 – Identification of Microalgal Species

Following the results and accomplishments are described for the three tasks of this project.

Task 1 - Sampling

For sampling a variety of aqueous habitats was chosen to increase the diversity of microalgae that could be isolated. It can be expected that microalgae thriving not only in extreme conditions, but also in habitats that are subject to drastic changes in environments such as irradiance, temperature, and salinity have a very versatile metabolism. Sampling sites were chosen to represent a variety of often very shallow aqueous habitats of one to five inches depth that were expected to be subject to large short term-changes in irradiance and temperature.
Habitats from which water samples were taken fell into the following major categories:

A. Freshwater
    a. Lakes
    b. Ponds
    c. Birdbaths
    d. Rivers
    e. Creeks
B. Brackish
    a. Estuaries, seawater-based
    b. Inland estuaries, non-seawater based
C. Marine
D. Hypersaline
    a. ponds
    b. lakes

Three major sampling locations were included for this project:
1. The variety of aqueous habitats in Brooklyn.
2. The Great Salt Lake in Utah and adjacent areas.
3. The Salton Sea in Southern California.

Some additional sites were located in Nevada and Northern California. An overview of the three major sampling sites is provided by Figures 1 to 3.

Figure 1: Shown are aerial photos taken from Google Earth to indicate exemplary sampling sites within Brooklyn (yellow arrows).

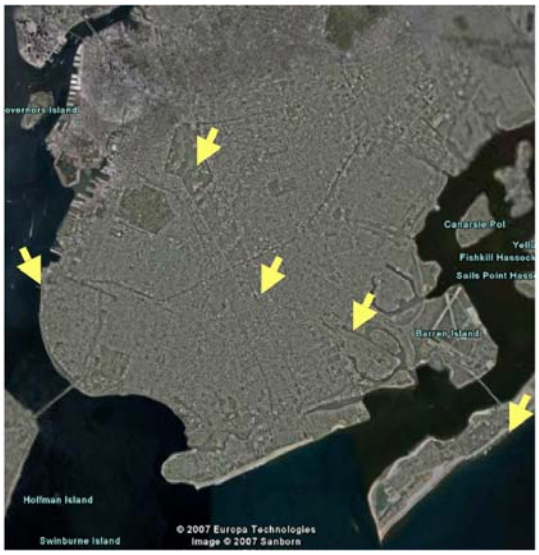


Figure 2: The Great Salt Lake in Utah as one major sampling location. A Google Earth photo shows the salt lake with several different habitats within and around the lake. Exemplary sampling sites are marked by yellow pins.

Figure 3: Shown is an aerial photo of the Salton Sea taken from Google Earth. Various sampling sites are indicated as yellow pins.

A number of different sampling sites was used throughout the borough of Brooklyn. Sampling was performed by Dr. Polle and also by several high-school students. Habitats from Brooklyn included freshwater, brackish, and marine water bodies.

A total of two sampling excursions were made to the Great Salt Lake. In addition, water samples were obtained directly from collaborators. The first excursion fell into the time that algal blooms were reported in the lake in late June 2007. The second sampling excursion was in mid September. Samples were taken within the salt lake itself at a number of sites. Further, samples were taken at various sites around the lake. Sampling sites included small to large ponds of various salinities. For example, in September 2007 one sampling location included the hypersaline salterns of the company Cargill located in the Southwest of the lake. Although all hypersaline, the ponds at Cargill had each very different salt compositions thus representing unique and extreme habitats. Also one saline hotspring, with a salinity of about 3% and a water temperature of about 42 Degree Celsius, which is located in the northwest of the Great Salt Lake was included as one sampling site.

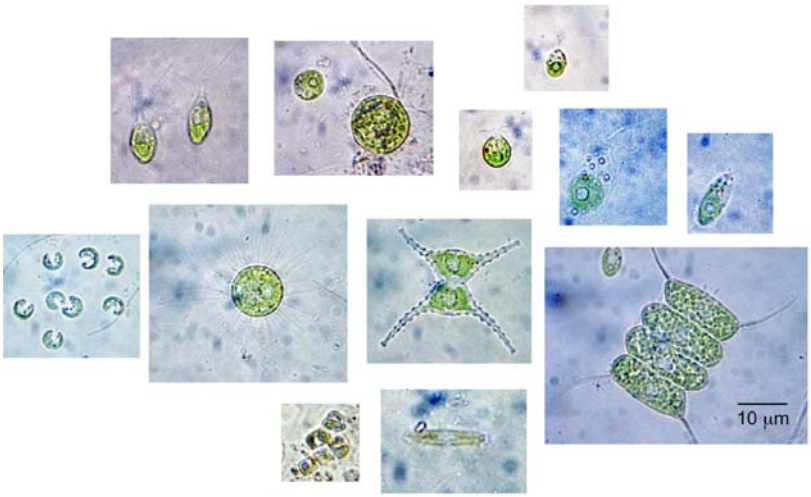
Sampling at the great Salt Lake was performed in collaboration with Dr. B. Baxter from Westminster College, the Great Salt Lake Ecosystem Program, and the AFOSR Biosolar group members.

One sampling excursions was made to the Salton Sea in Southern California in December 2007. Some samples were taken directly from the Salton Sea which has a salinity of about 4% for example at the Sonny Bono Reserve. Other samples were taken close to the lake for example from canals and estuaries. Based on microscopic investigation some of these samples contained a variety of diatoms.

Isolation of Strains
Focus of isolation of strains was on green algae and diatoms. However, any algae that came up in the media applied for growth was isolated. Thus, for example strains of Cyanobacteria (Bluegreen Algae) and Euglenoids were isolated.

In general, water samples were investigated by microscopy to determine how many different microalgae species were present in the sample. Figure 4 shows as an example the diversity of microalgae that was present in a small freshwater fish pond. From this microscopic investigation it was expected that about 3 to 10 different types of microalgae would be isolated from each sample. However, it turned out that this was not always the case, because not all microalgae strains would grow in the media employed.

Figure 4: Photographs of exemplary microalgal cells from a small fish pond. For example, several Desmids and Chlamydomonads were identified.



The following categories and media types were used for isolation of microalgal strains:

A. Freshwater
   Bold Basal Medium (BBM), Clostridium Medium (CM), BG11, High Salt Medium (HS), Tris Acetate Phosphate medium (TAP), filtered pond water (JPG)
B. Brackish
   Cyclotella Medium
C. Artificial seawater Medium (ASM)
D. Dunaliella Medium (DM)

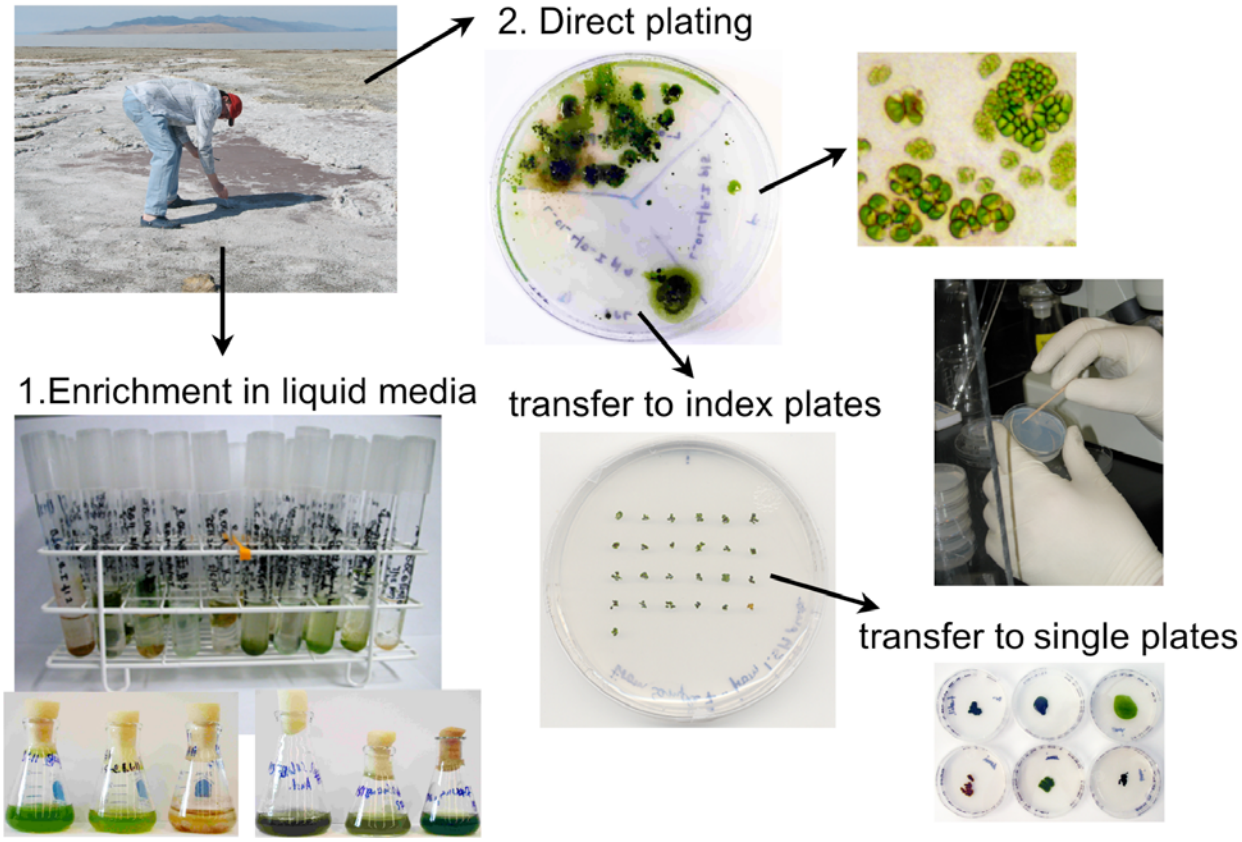
Notes about the media used:

- All media were made without addition of vitamins, because it was assumed that addition of vitamins to microalgae that will be cultivated on a very large scale will not be feasible.
- The majority of media was made only with minerals for photoautotrophic growth of cells.
- The Bold Basal Medium is well known for cultivation of a variety of green algae.
- The medium for Clostridium cultivation is known to support growth also of a variety of other green algae.
- The medium BG11 is a medium known to work well for growth of cyanobacteria.

- The so called 'high salt' medium is still a freshwater medium known to support growth of Chlamydomonads.
- TAP medium contains acetate as a carbon source for photoheterotrophic growth of cells.
- All media were made without and with supplemented silicate to allow growth of diatoms.
- Pond water was sterilized by filtration and mixed with other defined media to increase the variety of media available for cultivation.

For isolation of novel microalgal strains traditional enrichment and plating techniques were used. Figure 5 summarizes the process of enrichment and isolation.

Figure 5: Shown is the process of isolation of microalgae using either liquid enrichment cultures or agarose plates.



Identification of Microalgal Species

Newly isolated unialgal strains were kept on agarose plates or in liquid medium. Following isolation, identification of unialgal strains was routinely performed by microscopy. The following morphological markers were used for identification of species:

- Cell size & cellular shape
- Cell wall
- Flagella (absence, number)
- Eye spot (size, number)
- Pyrenoid

In addition to identification of novel strains by microscopy, from a variety of strains DNA was isolated using the MoBio Plant DNA isolation kit. Standard PCR was performed with general primers against the 18s rDNA gene or the rDNA internal transcribed spacer region 2 (ITS2). These nuclear marker genes were chosen for species identification, because a large number of sequences was available from NCBI for comparison. Following PCR products were isolated by standard agarose gel electrophoresis, gel extracted using the QUIAGEN gel extraction kit, and send out for sequencing. Following sequencing, the obtained sequences were blasted in NCBI for identification of sequences of similar organisms. Alignment of sequences was performed using the MegAlign program of DNASTAR and preliminary phylogenetic trees were generated using the same program. Based on sequence similarity of the markers 18s rDNA and ITS2 rDNA sequences novel strains were identified. An exemplary phylogram is shown in Figure 7 for strains of the genus *Dunaliella* isolated from the Great Salt Lake.

Figure 6: Identification of some exemplary species of green algae by microscopy. Upperleft – Gloeocystis, upper right – Chlorella, lower left – Scenedesmus, lower center – Fragillaria, lower right – Chlamydomonad.

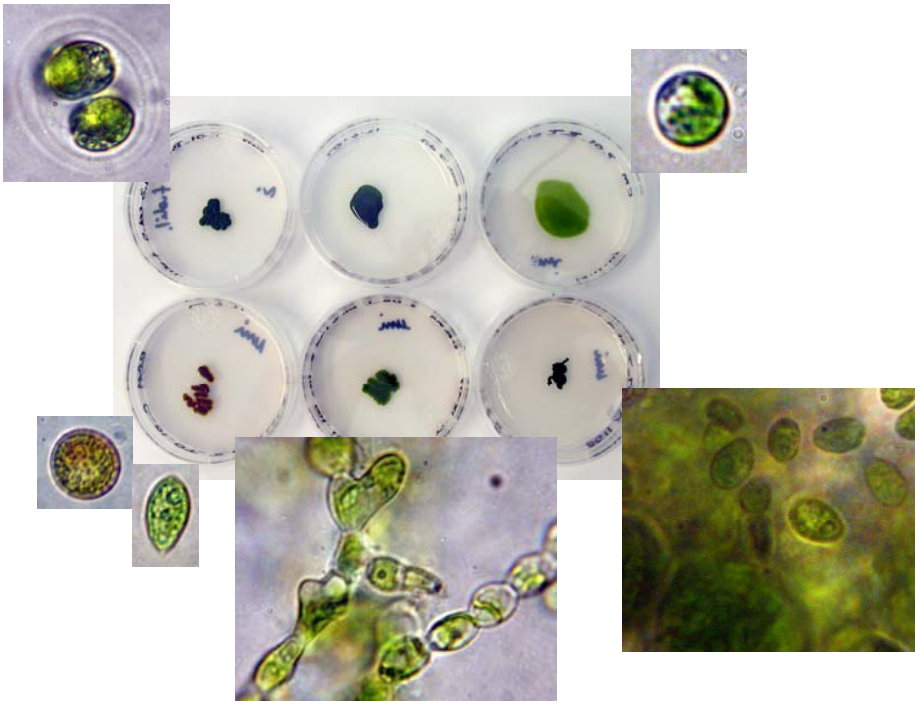
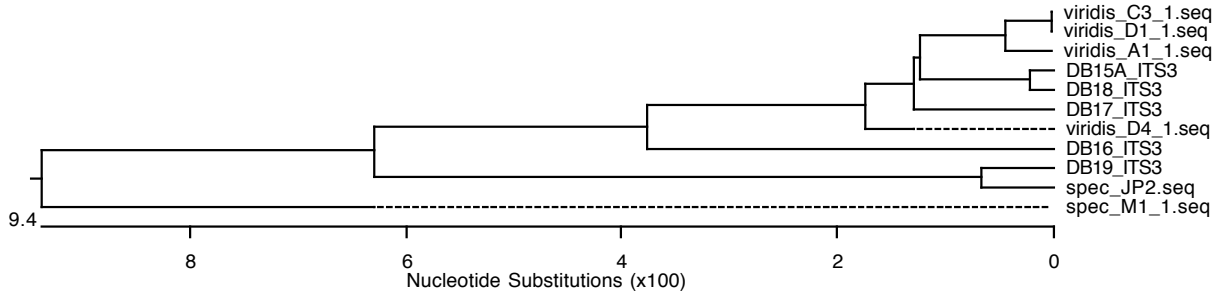


Figure 7: Shown is a phylogram based on the nuclear marker ITS2 for isolates of the species Dunaliella.



III. DISCUSSION

Following the suggestions of the proposal reviewer's and as an outcome of the first meeting of the microalgae group in August 2007 in Arlington, the primary focus of the project was adjusted to concentrate on the very time consuming isolation efforts for being able to find the larges number of novel microalgal strains possible. In order to be able and isolate more strains, the work effort for the originally proposed tasks of 'Creation of a Database' was reduced to assembly of data on sampling locations into EXCEL spreadsheets. Also, following the reviewer's suggestion, the task of 'Testing for Genes of Key Enzymes of Metabolic Pathways' was postponed to a later time following the first screening.
From freshwater samples the portion of isolated strains belonging to the green algae within the group of Demids was greatest. From a total of about 150 strains about 1/3 were a variety of desmids including but not limited to *Scenedesmus*, *Tetradesmus*, *Chlamydomonas*, *Chlorella*, *Chlorococcum*, *Haematococcus*, and *Kirchneriella*. Identification was not always possible only based on morphological markers such as the following. Overall, about 20% of the strains isolated from freshwater habitats were *Scenedesmus* (Chlorophyte) species. It was noted that *Scenedesmus* isolates grew very fast. They were often the first colonies to visibly appear on plates. This was not unexpected, because in outdoor open pond and also closed photobioreactor mass cultures strains of fast growing *Scenedesmus* species were known to be one of the major competitors to the cultured strains such as *Heamatococcus*.

On the other hand strains of the Chlorophyte genus *Dunaliella* constituted the largest portion of strains isolated from hypersaline habitats with salinities of more than 10%. Based on analysis of the nuclear molecular marker ITS2 it was revealed that a great diversity exists in the different dunaliella strains isolated from the Great Salt Lake. For example, it was discovered that at least two previously undescribed species of Dunaliella exist at the Great Salt Lake. Also, there appears to exist great diversity even within the isolates of a single species such as *Dunaliella viridis*.

A number of novel strain were isolated that appear to over-accumulate carotenoids under some growth conditions. According to literature, carotenoid accumulation in algal cells relies on concomitant lipid accumulation.

Sampling had to be accompanied by the process of obtaining permits for taking water samples. In many cases where the sampling site was located on private property permission to sample was obtained from the property owner. Currently, only oral permission exists. However, if any strain from these sources will be identified as useful for potential application in production of jetfuel, it is possible to obtain written permits. In order to reduce the time for obtaining permits, samples from the Great Salt Lake and from sites within Brooklyn were taken under the permit of collaborators. For sites at the Salton Sea in California a permit was obtained directly from the responsible Park Service.

In summary, the goal to isolate about 150 novel microalgal strains was achieved. At this time the project continues with a new grant from the AFOSR to isolate more strains and at the same time screen the existing collection for lipid production capabilities.